

# Analogues of the dihydroceramide desaturase inhibitor GT11 modified at the amide function: synthesis and biological activities†

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Dihydroceramide desaturase is the last enzyme in the biosynthesis of ceramide *de novo*. The cyclopropene-containing sphingolipid GT11 is a competitive inhibitor of dihydroceramide desaturase. The biological effects of chemical modification of the GT11 amide linkage are reported in this article. Either *N*-methyl substitution or replacement of the amide  $\alpha$ -carbonyl methylene by oxygen result in inactive compounds. In contrast, both urea (**3**) and thiourea (**4**) analogues of GT11, as well as three  $\alpha$ -ketoamides (**5–7**), did inhibit the desaturation of *N*-octanoylsphinganine to *N*-octanoylsphingosine, although with significantly lower potency than GT11. Furthermore, the  $\alpha$ -ketoamides **5–7** inhibit the acidic ceramidase with similar potencies ( $IC_{50}$  52–83  $\mu$ M). Inhibition of the neutral/alkaline ceramidase by these compounds requires around 20-fold higher concentrations. Structure–activity relationships and the biological interest of these compounds are discussed.

## Introduction

Besides its essential roles in cell biology, ceramide is the central molecule in the biosynthesis of sphingolipids and glycosphingolipids, which are also crucial in regulating various cell functions.<sup>1</sup> *De novo* biosynthesis of ceramide occurs from L-serine,<sup>2</sup> which is condensed with palmitoyl-CoA to give 3-ketosphinganine. Further reduction and subsequent *N*-acylation afford dihydroceramide, which is finally converted into ceramide by dihydroceramide desaturase.<sup>3</sup> Two different dihydroceramide desaturases have been so far reported, namely DES1,<sup>4</sup> which exhibits delta-4 desaturase activity, and DES2,<sup>5</sup> which has bifunctional delta-4 desaturase and hydroxylase activities. The latter is especially abundant in skin, intestines, and kidney, sites reportedly possessing high levels of phytosphingolipids.<sup>5</sup> Catabolism of ceramide occurs by the action of ceramidases, which hydrolyze the amide bond to yield the sphingosine moiety and fatty acids. According to their pH optima for activity, ceramidases fall into three groups, acidic, neutral and alkaline. The acid ceramidase is localized in the lysosomes and its defect causes Farber's disease.<sup>6</sup> Neutral ceramidases have been reported in several species<sup>7</sup> and they are localized in the endoplasmic reticulum and the mitochondria. Finally, alkaline ceramidases have also been found in guinea pig,<sup>8</sup> human skin,<sup>9</sup> bacteria<sup>10</sup> and yeast.<sup>11</sup>

The cyclopropene-containing sphingolipid GT11 is a competitive inhibitor of dihydroceramide desaturase with a  $K_i$  of 6  $\mu$ M.<sup>12</sup> Structure–activity correlations showed that the presence of a cyclopropene ring in place of the ceramide double bond, the natural 2*S*,3*R* stereochemistry, and a free hydroxyl group at C1 are required for inhibition.<sup>12</sup> Although it was also shown that a similar *N*-acyl chain length in both substrate and cyclopropene inhibitor were essential for inhibitory activity *in vitro*,<sup>12</sup> modifications of the GT11 amide linkage other than the *N*-acyl chain length were not performed. In light of several articles reporting on substrate based inhibitors of mitochondrial ceramidase<sup>13</sup> and fatty acid amide hydrolases,<sup>14–16</sup> such modifications might afford GT11 analogues with either higher

metabolic stability toward ceramidases or additional activity as ceramidase inhibitors. Both metabolically stable and ceramidase inhibitor analogues of GT11 would be of interest, since GT11 is partly degraded by ceramidases in cultured cells.<sup>17</sup> In this article we describe the synthesis of several analogues of GT11 (Fig. 1), and their *in vitro* activity on dihydroceramide desaturase and both the neutral/alkaline and the acidic ceramidase.

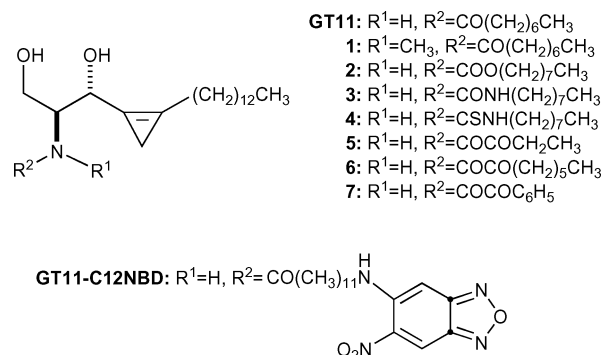


Fig. 1 Compounds used in this study.

## Results

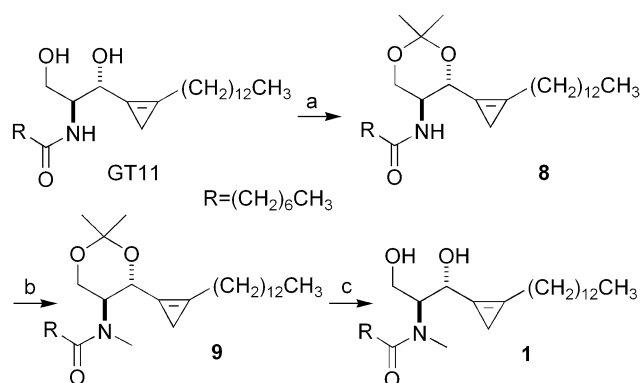
### Synthesis of GT11 analogues

The *N*-methylamide derivative **1** was obtained from GT11<sup>12</sup> through a protection–deprotection sequence (Scheme 1). Thus, GT11 was transformed into the dioxolane **8** under standard conditions. Treatment of **8** with NaH and further reaction with MeI afforded **9**, which gave rise to the expected analogue **1** upon mild acid hydrolysis.

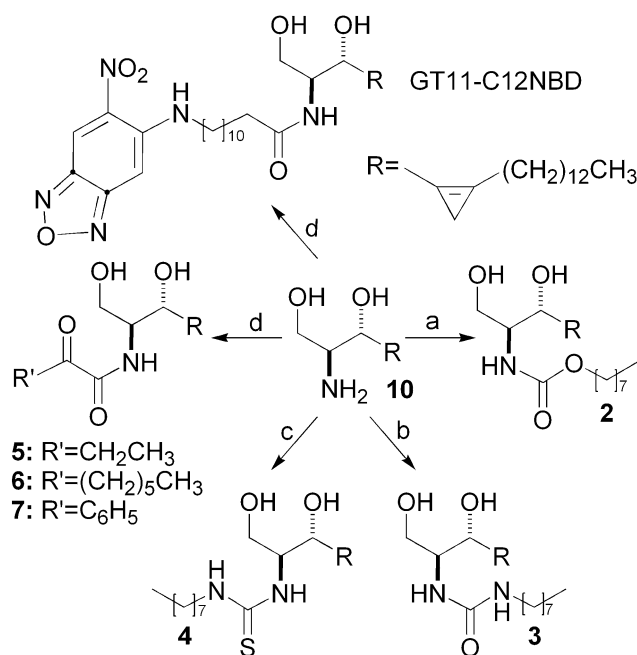
The carbamate **2** and urea **3** were obtained in moderate yields by reaction of the aminodiol **10**<sup>18</sup> with octyl chloroformate and octyl isocyanate, respectively (Scheme 2). In the synthesis of **2**, reaction until complete consumption of the starting material afforded mixtures of the expected carbamate along with two other carbamates having each of the two hydroxyl groups esterified with octylcarboxylic acid. Careful monitoring of the reaction course by thin layer chromatography and its immediate quenching as soon as carbonates were noticeable,

† Dedicated to Professor Francesc Camps on occasion of his 70th birthday.

‡ Both authors contributed equally to this work.



**Scheme 1** Synthesis of **1**. a, 2,2-dimethoxypropane, pyridinium *p*-toluenesulfonate,  $\text{CH}_2\text{Cl}_2$ ; b, sodium hydride, dimethyl sulfoxide, then methyl iodide in tetrahydrofuran; c, *p*-toluenesulfonic acid in methanol (40% overall yield from **8**).



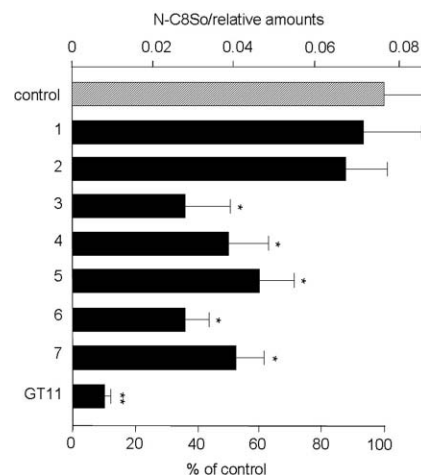
**Scheme 2** Synthesis of **2–7** and GT11-C12NBD. a,  $\text{CH}_3(\text{CH}_2)_7\text{CO}_2\text{Cl}$  in methylene chloride–triethylamine; b,  $\text{CH}_3(\text{CH}_2)_7\text{NCO}$  in diethyl ether–acetonitrile; c,  $\text{CH}_3(\text{CH}_2)_7\text{NCS}$  in diethyl ether–acetonitrile; d, treatment with the appropriate acid in the presence of EDC–HOBT in either methylene chloride or diethyl ether–acetonitrile. Acids are NBD( $\text{CH}_2$ )<sub>11</sub>COOH (GT11-C12NBD);  $\text{CH}_3\text{CH}_2\text{COCOOH}$  (**5**);  $\text{CH}_3(\text{CH}_2)_5\text{COCOOH}$  (**6**) and  $\text{C}_6\text{H}_5\text{COCOOH}$  (**7**).

even with remaining starting material, allowed the preparation of **2** in moderate yields (40%) in the absence of by-products. The thiourea **4** was prepared by reaction of aminodiol **10** with octyl isothiocyanate (Scheme 2). The  $\alpha$ -ketoamides **5–7** were synthesized from aminodiol **10** and the suitable  $\alpha$ -ketoacid in the presence of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC) and 1-hydroxybenzotriazole (HOBT) (Scheme 2). The same procedure was followed to prepare the fluorescent derivative of GT11 (GT11-C12NBD) from **10** and 12-(4-nitrobenzo-2-oxa-1,3-diazolo)dodecanoic acid (Scheme 2).

### Biological activities of the GT11 analogues

**Inhibition of dihydroceramide desaturase.** The effect of the cyclopropeneceramides **1–7** on dihydroceramide desaturase was investigated using rat liver microsomes and the previously reported assay.<sup>12</sup> Neither *N*-methylamide **1** nor carbamate **2** inhibited dihydroceramide desaturase. In contrast, both urea **3** and thiourea **4**, as well as the three  $\alpha$ -ketoamides **5–7** inhibited the desaturation of *N*-octanoylsphinganine to *N*-octanoylsphingosine (Fig. 2), with inhibition percentages rang-

ing from 40 to 70% at equimolar concentrations of substrate and inhibitor (50  $\mu\text{M}$ ). In the same experimental conditions, GT11 caused a 91% inhibition and was significantly more potent than the other **5** compounds.



**Fig. 2** Effect of compounds GT11 and **1–7** on dihydroceramide desaturase. The experiment was performed as detailed in ref. 21. The amounts of *N*-octanoylsphingosine (NC8So) were calculated by gas chromatography coupled to mass spectrometry relative to a known amount of *N*-hexanoylsphingosine used as internal standard. The characteristic ions at  $m/z$  258 and 230, corresponding to the trimethylsilyl derivatives of *N*-octanoylsphingosine and *N*-hexanoylsphingosine, respectively, were used for quantification. Data correspond to the mean  $\pm$  standard deviation of three replicates. Asterisks indicate statistical significance (unpaired two-tail *t* test, \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.005$ ).

**Ceramidase activity.** The effect of the dihydroceramide desaturase inhibitors **3–7** on ceramidase activities was determined by quantification of the fluorescent fatty acid released from 12-(4-nitrobenzo-2-oxa-1,3-diazolo)dodecylsphingosine (Cer-C12NBD). While in previously reported assays<sup>19</sup> both substrate and acid were separated by HPLC, a modification has been introduced here consisting of the separation of both fluorescent species by ion exchange chromatography, using a commercially available cation-exchange resin. Filtration of the reaction mixture through the resin and washing with buffer-methanol at  $\text{pH} \geq 7.5$  results in the elution of the unreacted substrate and retention of the enzymatically released acid, which is then eluted with a mixture of acetic acid–methanol. Aliquots of the several fractions are finally transferred to 96-well plates, the fluorescence is determined and the actual amounts of both substrate and product are calculated from standard curves. This assay was validated by HPLC, which clearly showed that the neutral eluates filtered through the column contained only the Cer-C12NBD substrate, whereas the acidic eluates contained exclusively the fluorescent acid (data not shown). In our conditions, hydrolysis of Cer-C12NBD by the neutral ceramidase preparation occurred with an apparent  $K_m$  of 106.7  $\mu\text{M}$  and a  $V_{\text{max}}$  of 17.3  $\text{pmol min}^{-1} \text{mg}^{-1}$  ( $V_{\text{max}}/K_m = 0.162$ ). The acidic ceramidase hydrolyzed Cer-C12NBD with a  $K_m$  of 64.5  $\mu\text{M}$  and a  $V_{\text{max}}$  of 11.3  $\text{pmol min}^{-1} \text{mg}^{-1}$  ( $V_{\text{max}}/K_m = 0.175$ ).

To investigate the hydrolysis of GT11 by ceramidases, the fluorescent compound GT11-C12NBD, which was used as substrate, was incubated with the suitable enzyme preparation and the released fluorescent fatty acid was quantified after ion exchange chromatography of the incubation mixture. This procedure evidenced that GT11-C12NBD was hydrolyzed by the neutral ceramidase with a  $K_m$  of 85.2  $\mu\text{M}$  and a  $V_{\text{max}}$  of 9.4  $\text{pmol/min/mg}$  ( $V_{\text{max}}/K_m = 0.110$ ) and by the acidic ceramidase with a  $K_m$  of 69.2  $\mu\text{M}$  and a  $V_{\text{max}}$  of 6.9  $\text{pmol min}^{-1} \text{mg}^{-1}$  ( $V_{\text{max}}/K_m = 0.100$ ).

**Inhibition of ceramidases.** None of the dihydroceramide desaturase inhibitors **3–7** inhibited the neutral ceramidase at

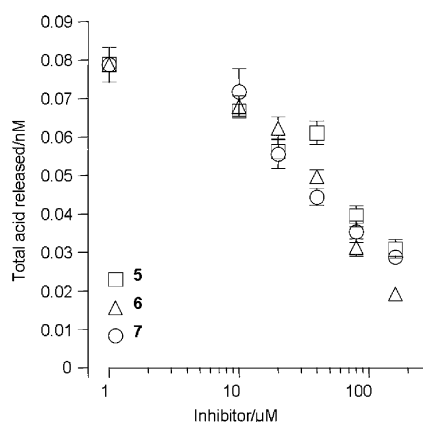
**Table 1** Effect of GT11 analogs on the neutral ceramidase

	Control	3	4	5
Total acid released/nM	305 ± 6	80 ± 84	41 ± 22	120 ± 28
% Inhibition		74	86	61

<sup>a</sup> Enzyme activity was determined as indicated in the Experimental section with 300 µg of protein using a substrate concentration of 25 µM and a 500 µM concentration of inhibitors. The amounts of acid released after a 1 h incubation were determined after ion exchange chromatography. Data correspond to the mean ± standard deviation of four replicates. % Inhibition was calculated using the equation  $100(\text{acid}_{\text{control}} - \text{acid}_{\text{inhibitor}} / \text{acid}_{\text{control}})$ , where  $\text{acid}_{\text{control}}$  and  $\text{acid}_{\text{inhibitor}}$  correspond to the total amounts of acid released from mitochondria incubated in the absence (untreated control) or the presence of inhibitors, respectively.

equimolar concentrations (25 µM) with respect to the Cer-C12NBD substrate, although they were presumably stable towards deacylation.<sup>13</sup> A 74% inhibition of the neutral ceramidase was produced with 500 µM of the urea derivative **3** (substrate : inhibitor molar ratio 1 : 20). In the same experimental conditions, the thiourea **4** and ketoamide **5** produced 86% and 61% inhibition, respectively (Table 1).

The GT11 analogs **3** and **4** were not inhibitors of the acid ceramidase at equimolar concentrations with respect to the substrate (40 µM). However, the  $\alpha$ -ketoamides **5–7** did inhibit this enzyme and the effect was concentration-dependent (Fig. 3). The  $\text{IC}_{50}$  values obtained at a single substrate concentration (40 µM) indicated that the three compounds **5–7** had similar potencies ( $\text{IC}_{50}$  52–83 µM). Although kinetic studies were conducted, the type of inhibition could not be ascertained, probably because a non-purified homogenate was used as the source of enzyme.



**Fig. 3** Dose-response of  $\alpha$ -ketoamides **5–7** on the acid ceramidase. Enzyme activity was determined as indicated in the experimental section using a substrate concentration of 40 µM and different concentrations of inhibitors (10, 20, 40, 80 and 160 µM). Data correspond to the mean ± standard deviation of a representative experiment with three replicates. The  $\text{IC}_{50}$  values calculated on the basis of a regression analysis were: **5**, 83 µM; **6**, 61 µM; **7**, 52 µM.

## Discussion

Dihydroceramide desaturase is the last enzyme in the *de novo* biosynthetic pathway of ceramide.<sup>3</sup> Two different dihydroceramide desaturases, DES1<sup>4</sup> and DES2,<sup>5</sup> have so far been reported. Although their amino acid sequences have high identity, they differ in their enzymatic characteristics, since while DES1 exhibits high dihydroceramide desaturase activity and very low C-4 hydroxylase activity, DES2 is similarly active as both a dihydroceramide desaturase and a C-4 hydroxylase. In previous articles we reported that the cyclopropene-containing sphingolipid GT11 is a competitive inhibitor of dihydroceramide

desaturase.<sup>12,18</sup> In structure–activity correlation studies<sup>18</sup> we found that the natural 2*S*,3*R* stereochemistry, the amide function, the presence of a cyclopropene ring in place of the ceramide double bond and a free hydroxyl group at C1 are required for inhibition. Additional structure–activity correlations are provided in this article, in which we report that *N*-methyl substitution of GT11 results in the loss of inhibitory activity. Although the steric hindrance of the methyl group can be invoked to explain the lack of effect of **1**, it is also possible that the amide hydrogen atom, which is absent in the *N*-methylamide **1**, is essential for inhibitory activity by virtue of its participation in hydrogen bonding events required for inhibition, (*i.e.* enzyme–inhibitor interactions or arrangement of the inhibitor into the active conformation).

Interference with hydrogen bonding can also be a reason for the ineffectiveness of carbamate **2** on dihydroceramide desaturase, in contrast to the respective urea **3** and thiourea **4** analogs, which retain inhibitory activity. By means of its higher hydrogen bond accepting character as compared to the urea **3** and thiourea **4** amino counterparts, the carbamate oxygen of **2** may compete and thus interfere with hydrogen bonding necessary for inhibition. Since the  $\alpha$ -ketoamides **5–7** retained dihydroceramide desaturase inhibitory activity, these overall results indicate that some substitutions are allowed in the  $\alpha$  position of the amide carbonyl moiety of GT11, although they result in less potent inhibitors than the lead compound.

Besides providing with additional structure–dihydroceramide desaturase inhibitory activity correlations, the activity of some GT11 analogs on ceramidases deserves attention. The urea isostere of *N*-palmitoylsphingosine was reported as a competitive inhibitor of the rat brain neutral ceramidase with a  $\text{IC}_{50}$  of 25 µM at 50 µM concentration of substrate (Cer-C12NBD).<sup>13</sup> In contrast, the urea derivative of GT11 (**2**) had no effect on the neutral ceramidase at equimolar concentrations with respect to the substrate and high inhibitor : substrate molar ratios were required for inhibition. This was also the case for the thiourea isostere **4**. Considering that *N*-acyl sphingosines with acyl chain lengths below C10 are poor substrates of the neutral ceramidase,<sup>20</sup> it is possible that the short *N*-acyl chain of the urea analog **3** (and thiourea **4**) lowers the affinity of the enzyme for this compound, which would result in its decreased potency as compared to the *N*-palmitoylsphingosine urea analog. It has been shown that the neutral ceramidase from rat brain hydrolyzes dihydroceramide at a 10-fold lower rate than ceramide and that the *cis* analog of ceramide is not hydrolyzed.<sup>20</sup> Taking into account this substrate specificity, one could argue that the presence of a cyclopropene ring in **3** (and **4**) diminishes the enzyme affinity for this compound, which is significantly less potent than the *N*-palmitoylsphingosine urea analog. However, the finding that Cer-C12NBD and GT11-C12NBD are hydrolyzed by the neutral ceramidase with similar kinetic parameters seems to indicate that the cyclopropene ring does not affect enzyme binding and therefore, the first explanation invoking the *N*-acyl chain length seems more plausible.

Although several authors have reported that  $\alpha$ -ketoamides are competitive inhibitors of serine hydrolases, including fatty acid amide hydrolases,<sup>14–16</sup> the structural requirements of the neutral ceramidase<sup>20</sup> is also a likely explanation for the low effectiveness of the  $\alpha$ -ketoamides **5–7** on this enzyme. Therefore, despite the occurrence of the  $\alpha$ -ketoamide unit, sphingoid base acylation with short  $\alpha$ -ketoamide units lower the affinity of the enzyme for these compounds.

The GT11 analogs **3** and **4** did not inhibit the acidic ceramidase. In contrast,  $\alpha$ -ketoamides **5–7** inhibited this enzyme in a concentration-dependent manner with similar potencies ( $\text{IC}_{50}$  52–83 µM). As reported for  $\alpha$ -ketoamide inhibitors of fatty acid amide hydrolases,<sup>14</sup> the electron-deficient  $\alpha$ -carbonyl group does probably react with the active-site nucleophilic amino acid to form a hemiacetal intermediate, which mimics the reaction



transition state thus inhibiting the hydrolysis. The additional features defined in the different  $\alpha$ -ketoamides may contribute independently to enzyme binding affinity. Although the activity of the acid ceramidase in the presence of different amounts of inhibitor while varying the substrate concentration was investigated, no conclusive data as to the type of inhibition could be obtained, probably because a non-purified lysosomal fraction was used as the source of enzyme. Taking into account the results reported for  $\alpha$ -ketoamide inhibitors of amide hydrolases,<sup>16</sup> a competitive type of inhibition would be expected for compounds **5–7**.

In a previous work<sup>17</sup> we showed that in primary cultured neurons, GT11 inhibited dihydroceramide desaturase in a concentration dependent manner ( $IC_{50}$  of 23 nM) with a potency by three orders of magnitude higher than that found *in vitro*. However, although this inhibitory effect was specific at concentrations up to 1  $\mu$ M, higher concentrations (from 5  $\mu$ M upwards) of GT11 not only abolished desaturation of dihydroceramide, but also impaired *de novo* sphingolipid biosynthesis by an indirect suppression of serine palmitoyltransferase activity. Metabolic studies with radioactively labeled GT11 analogs indicated that the inhibitor is subjected to catabolism by ceramidases to yield the cyclopropene-bearing long chain base. In this study we show that *in vitro* *N*-deacylation of GT11 is produced by both the acidic and the neutral ceramidase. Although the free cyclopropene base does not inhibit dihydroceramide desaturase,<sup>18</sup> it cannot be completely disregarded that deacylated GT11 or its phosphate are involved in the effect of high concentrations of GT11. The study of the activities of the GT11 analogs reported here in cell culture should help clarify the mechanism underlying the effects of high concentrations of GT11 in cultured cells.

Several inhibitors of the neutral/alkaline,<sup>13,21,22</sup> and the acidic ceramidase<sup>22,23</sup> have been reported. The latter include *N*-oleylethanolamine, which also affects other amide hydrolases<sup>24</sup> and has a rather low potency, (1*R*,2*R*)-2-(*N*-tetradecylamino)-1-(4-nitrophenyl)-1,3-propanediol (AD2646)<sup>25</sup> and (1*R*,2*R*)-2-(*N*-tetradecanoylamino)-1-(4-nitrophenyl)-1,3-propanediol (B-13).<sup>22,23</sup> The latter two compounds suppress acid ceramidase activity with good potencies and the activity of the neutral/alkaline isoform is affected only slightly. The mechanism of inhibition by these compounds is unknown. In this article we report the first family of mechanism-based inhibitors of the acid ceramidase with potencies in the low  $\mu$ M range in an acidic ceramidase preparation. Remarkably, these compounds have a good selectivity toward of the neutral ceramidase *in vitro*, which requires around 20 fold higher concentrations for inhibition. Since  $\alpha$ -ketoamides **5–7** have dual inhibitory activity on both dihydroceramide desaturase and the acidic ceramidase, they should induce an accumulation of dihydroceramide in cultured cells. Therefore, this family should prove very useful to evaluate the role of dihydroceramide in cell biology, a matter of current debate. Whereas the cyclopropene ring is crucial for dihydroceramide desaturase inhibition,<sup>18</sup> it is not likely essential for inhibition of the acidic ceramidase. By similarity to other serine or cysteine hydrolases, the later activity does probably lie in the  $\alpha$ -ketoamide unit, by virtue of which these molecules behave as reversible transition state mimic inhibitors.<sup>14</sup> Thus, we anticipate that  $\alpha$ -ketoamides of sphingoid bases are attractive candidates as leads in the search for potent and specific ceramidase inhibitors. Work along this line is ongoing in our laboratories.

## Conclusions

In conclusion, either *N*-methyl substitution or replacement of the amide  $\alpha$ -carbonyl methylene by oxygen in the dihydroceramide desaturase inhibitor GT11 result in inactive compounds, whereas activity is retained in urea (**3**), thiourea (**4**) and  $\alpha$ -ketoamide (**5–7**) analogs of GT11. This difference can be explained considering that the inactive compound cannot

comply with hydrogen bonding events required for inhibition. Furthermore, the  $\alpha$ -ketoamides **5–7** have been identified as the first mechanism-based inhibitors of the acid ceramidase with potencies in the low  $\mu$ M range. Since  $\alpha$ -ketoamides **5–7** have dual inhibitory activity on both dihydroceramide desaturase and the acidic ceramidase, they should induce an accumulation of dihydroceramide in cultured cells. Therefore, these compounds should prove very useful to decipher the role of dihydroceramide in cell biology.

## Experimental

### Synthesis of compounds

**(4*R*,5*S*)-*N*-[2,2-Dimethyl-4-(2-tridecylcyclopropen-1-yl)-1,3-dioxan-5-yl]octanamide (**8**).** A solution of GT11 (50 mg, 0.11 mmol), pyridinium *p*-toluenesulfonate (5.7 mg, 0.023 mmol) and 2,2-dimethoxypropane (0.280 ml, 2.28 mmol) in  $CH_2Cl_2$  (5 ml) was stirred at room temperature for 12 h. After this time, the solvent was removed, the residue was dissolved in ethyl acetate and the organic solution was sequentially washed with saturated solution of  $NaHCO_3$  and brine. Solvent removal under reduced pressure afforded a reaction mixture that was purified by silica gel column chromatography ( $CH_2Cl_2$ –MeOH 98 : 2) to furnish 36 mg (0.075 mmol, 66%) of amide **8**. <sup>1</sup>H-NMR (300 MHz,  $CDCl_3$ ): 0.87 (t, 6H, *J* = 6.6 Hz), 1.01 (s, 2H), 1.2–1.4 (m, 28H), 1.58 (m, 4H), 2.17 (t, *J* = 7.5 Hz, 2H), 2.43 (t, *J* = 7.5 Hz, 2H), 3.68 (m, 1H), 4.07 (m, 2H), 4.69 (d, *J* = 6.5 Hz, 1H), 5.84 (d, *J* = 8.0). <sup>13</sup>C-NMR (75 MHz,  $CDCl_3$ ): 8.2, 14.0, 14.1, 22.5, 22.6, 24.1, 25.3, 25.6, 25.9, 27.0, 29.0, 29.1, 29.3, 29.4, 29.5, 29.6, 31.65, 31.8, 36.8, 46.7, 62.0, 69.4, 98.8, 107.1, 116.0, 172.6.

**(4*R*,5*S*)-*N*-[2,2-Dimethyl-4-(2-tridecylcyclopropen-1-yl)-1,3-dioxan-5-yl]-*N*-methyloctanamide (**9**).** A solution of **8** (36 mg, 0.07 mmol) in anhydrous tetrahydrofuran (2 ml) and anhydrous dimethyl sulfoxide (2 ml) was successively treated under argon with NaH (3 mg, 0.07 mmol) and  $CH_3I$  (21.3 mg 0.15 mmol). The reaction was stirred at room temperature for 12 h and the product was extracted with diethyl ether. The organic layer was washed with brine and the solvent was removed under reduced pressure to afford a reaction mixture (22 mg, 0.045 mmol, 64%) that was used in the following reaction without purification. <sup>1</sup>H-NMR (300 MHz,  $CDCl_3$ ): 0.87 (t, *J* = 6.9 Hz, 6H), 1.01 (m, 1H), 1.2–1.4 (m, 28 H), 1.42 (d, *J* = 11.1 Hz, 3H), 1.55 (d, *J* = 7.8 Hz, 3H), 1.58 (m, 4H), 2.27 (m, 2H), 2.40 (t, 2H), 3.90 (s, 1.5H), 3.05 (s, 1.5H), 3.91 (m, 2H), 4.17 (m, 1H), 5.02 (d, *J* = 9 Hz, 1H), 5.84 (d, *J* = 6.6 Hz). <sup>13</sup>C-NMR (75 MHz,  $CDCl_3$ ): 7.6, 7.8, 14.1, 19.9, 22.6, 22.67, 25.2, 26.0, 27.1, 27.18, 27.6, 28.1, 28.5, 29.1, 29.3, 29.6, 31.7, 31.9, 33.2, 34.3, 37.0, 54.5, 59.8, 61.1, 65.0, 66.2, 99.1, 99.2, 105.6, 106.5, 116.1, 117.2, 173.2, 173.4.

**(1*S*,2*R*)-*N*-[2-Hydroxy-1-hydroxymethyl-2-(2-tridecylcycloprop-en-1-yl)ethyl]-*N*-methyloctanamide (**1**).** Crude amide **9** (20 mg, 0.040 mmol) and *p*-toluenesulfonic acid (3.25 mg, 0.02 mmol) were dissolved in MeOH (2 ml) and the solution was stirred at room temperature for 2 h. After this time, the solvent was evaporated and the residue was dissolved in ethyl acetate. The organic solution was successively washed with saturated solution of  $NaHCO_3$  and brine. The solvent was removed under reduced pressure to afford a reaction mixture that was purified by silica gel column chromatography ( $CH_2Cl_2$ –MeOH 98 : 2) to furnish 17 mg (0.038 mmol, 95%) of **1** (oil). [ $\alpha$ ]<sub>D</sub>: +1.03 (*c* 0.68,  $CHCl_3$ , 20 °C). <sup>1</sup>H-NMR (300 MHz,  $CDCl_3$ ): 0.87 (t, *J* = 6.3 Hz, 6H), 0.96 (AB system, *J* = 8.4 Hz, 2H), 1.01 (AB system, *J* = 8.4 Hz, 2H), 1.2–1.4 (m, 28H), 1.58 (m, 4H), 2.30 (t, *J* = 7.5 Hz 2H), 2.43 (dt, *J* = 7.2 and 1.2 Hz, 2H), 2.85 (s, 0.60H), 3.01 (s, 2.40H), 3.99 (m, 3H), 4.86 (d, *J* = 7.5 Hz, 0.60H), 5.06 (d, *J* = 5.7 Hz, 2.40H). <sup>13</sup>C-NMR (75 MHz,  $CDCl_3$ ): 8.0, 14.1, 22.6, 22.67, 24.9, 25.8, 27.2, 29.1, 29.3, 29.4, 29.47, 29.6, 31.6,

31.9, 34.4, 36.3, 61.6, 64.1, 67.8, 108.2, 114.2, 175.2. IR (film): 3397, 2926, 1623, 1463, 1034. ESI-MS:  $m/z$  452  $[M + 1]^+$ , 474  $[M + Na]^+$ . HR-MS calcd for  $C_{28}H_{53}NO_3$ : 451.40254; found: 451.40258.

**(1'S,2'R)-Octyl 2-hydroxy-1-hydroxymethyl-2-(2-tridecylcyclopropen-1-yl)ethyl]carbamate (2).** Triethylamine (11.40  $\mu$ L, 0.081 mmol) and octyl chloroformate (10.57  $\mu$ L, 0.054 mmol) was added at 0 °C to aminodiol **10** (17 mg, 0.054 mmol) dissolved in anhydrous  $CH_2Cl_2$  (3 mL). The mixture was stirred overnight at room temperature and the product was extracted with  $CH_2Cl_2$ . The organic layer was sequentially washed with 1 M HCl, saturated solution of  $NaHCO_3$  and brine. Solvent removal under vacuum afforded a reaction mixture that was purified by silica gel column chromatography (hexane–ethyl acetate 1 : 1) to give 10 mg (0.022 mmol, 41%) of **2**. Mp: 47 °C.  $[a]_D^{20}$ : +1.75 ( $c$  0.57,  $CHCl_3$ , 20 °C).  $^1H$ -NMR (300 MHz,  $CDCl_3$ ): 0.87 (t,  $J$  = 6.9 Hz, 6H), 1.01 (s, 2H), 1.2–1.4 (m, 32 H), 1.59 (m, 4H), 2.46 (dt,  $J$  = 7.5 and 1.5 Hz, 2H), 3.76 (m, 1H), 3.87 (m, 1H), 4.06 (t,  $J$  = 6.9 Hz, 2H), 4.86 (s, 1H), 5.51 (d,  $J$  = 8.4 Hz).  $^{13}C$ -NMR (75 MHz,  $CDCl_3$ ): 8.2, 14.1, 22.6, 22.6, 25.8, 27.2, 28.9, 29.1, 29.2, 29.3, 29.4, 29.5, 29.6, 31.7, 31.9, 54.7, 63.2, 65.4, 70.7, 107.5, 115.4, 156.9. IR (film): 3379, 2925, 1700, 1463, 1043.  $[a]_D^{20}$ : +1.87 ( $c$  0.88,  $CHCl_3$ , 20 °C). ESI-MS:  $m/z$  450  $[M + 1]^+$ , 490  $[M + Na]^+$ . HR-MS calcd for  $C_{28}H_{53}NO_4$ : 467.39746; found: 467.39749.

**(1'S,2'R)-1-[2-Hydroxy-1-hydroxymethyl-2-(2-tridecylcyclopropen-1-yl)ethyl]-3-octylurea (3).** A solution of aminodiol **10** (7 mg, 0.02 mmol) in  $CH_3CN$  (2.4 mL) and diethyl ether (1.4 mL) was reacted with octyl isocyanate (3.5  $\mu$ L, 0.02 mmol) at room temperature for 12 h. After this time, the solvent was removed under vacuum and the resulting residue was purified by silica gel column chromatography ( $CH_2Cl_2$ –MeOH 97 : 3) to furnish 7 mg (0.015 mmol, 75%) of the expected urea **3** (oil).  $^1H$ -NMR (300 MHz,  $CDCl_3$ ): 0.87 (t,  $J$  = 6.9 Hz, 6H), 1.00 (s, 2H), 1.2–1.4 (m, 28H), 1.52 (m, 4H), 2.45 (dt,  $J$  = 7.2 and 1.2 Hz, 2H), 3.14 (q,  $J$  = 6.9 and 2.6 Hz, 2H), 3.72 (dd,  $J$  = 3.3 and 11.1 Hz, 1H), 3.82 (dd,  $J$  = 4.5 Hz and 11.1 Hz, 1H), 4.04 (m, 3H), 4.62 (t,  $J$  = 4.5 Hz, 2H), 4.84 (s, 1H), 5.25 (d,  $J$  = 7.5 Hz).  $^{13}C$ -NMR (75 MHz,  $CDCl_3$ ): 8.3, 14.2, 22.8, 25.6, 26.9, 27.9, 29.2, 29.28, 29.5, 29.6, 30.0, 31.7, 31.9, 40.7, 54.8, 63.7, 70.8, 107.6, 115.2, 158.5. IR (film): 3335, 2924, 1628, 1463, 1021. ESI-MS:  $m/z$  467  $[M + 1]^+$ , 489  $[M + Na]^+$ . HR-MS calcd for  $C_{28}H_{54}N_2O_3$ : 466.41344; found: 466.41339.

**(1'S, 2'R)-1-[2-Hydroxy-1-hydroxymethyl-2-(2-tridecylcyclopropen-1-yl)octyl]-3-hexylthiourea (4).** Octyl isothiocyanate 20  $\mu$ L (0.12 mmol) was added to a solution of aminodiol **10** (37.3 mg, 0.12 mmol) in  $CH_3CN$  (2.4 mL) and diethyl ether (1.4 mL). After 72 h of stirring at 25 °C, the solvent was removed under reduced pressure and the resulting residue was purified by silica gel column chromatography. Elution with  $CH_2Cl_2$ –MeOH (97 : 3) gave 30 mg (0.066 mmol, 55%) of thiourea **4** (oil).  $^1H$ -NMR (300 MHz,  $CDCl_3$ ): 0.87 (t,  $J$  = 7 Hz, 6H), 1.04 (s, 2H), 1.2–1.4 (m, 28 H), 1.59 (m, 4H), 2.47 (dt, 2H,  $J$  = 7.5 and 1.5), 3.34 (bs, 2H), 3.85 (dd,  $J$  = 11.5 and 3.5 Hz), 3.94 (dd,  $J$  = 11 and 3 Hz, 1H), 4.6 (bs, 1H), 5.04 (s, 1H), 6.23 (d,  $J$  = 7.5 Hz, 1H), 6.57 (bs, 1H).  $^{13}C$ -NMR (75 MHz,  $CDCl_3$ ): 8.4, 13.9, 14.2, 22.5, 22.7, 25.9, 26.6, 27.3, 28.7, 29.3, 29.4, 29.46, 29.6, 29.66, 31.4, 31.9, 57.8, 63.3, 70.9, 107.3, 115.9, 181.4. IR (film): 3283, 2930, 1551, 1627, 1461, 1024. ESI-MS:  $m/z$  455  $[M + 1]^+$ , 477  $[M + Na]^+$ . HR-MS calcd for  $C_{28}H_{54}N_2O_2S$ : 482.39060; found: 482.39057.

**$\alpha$ -ketoamides.** To a solution of aminodiol **10** (0.1 mmol), EDC (0.15 mmol) and HOBt (0.15 mmol) was added the  $\alpha$ -ketoacid (0.15 mmol) dissolved in  $CH_2Cl_2$  (2 mL). The reaction mixture was stirred at 25 °C for 12 h,  $CH_2Cl_2$  (10 mL) was added and the organic phase was washed with brine and dried ( $MgSO_4$ ). The solvent was removed under vacuum and the resulting residue was purified by column chromatography on

silica gel eluting with a mixture of  $CH_2Cl_2$ –MeOH 97 : 3. Compound were obtained as colorless oils.

**(1'S,2'R)-N-[2-Hydroxy-1-hydroxymethyl-2-(2-tridecylcyclopropen-1-yl)ethyl]-2-oxobutanamide (5).** Yield 55% from 0.096 mmol of **10** and 2-oxobutanoic acid (0.11 mmol).  $[a]_D^{20}$ : –5.21 ( $c$  0.69,  $CHCl_3$ , 20 °C).  $^1H$ -NMR (300 MHz,  $CDCl_3$ ): 0.87 (t,  $J$  = 6.6 Hz, 3H), 1.03 (s, 2H), 1.12 (t,  $J$  = 6.9 Hz, 3H), 1.2–1.4 (m, 20H), 1.56 (t,  $J$  = 7.2 Hz, 2H), 2.46 (dt,  $J$  = 1.5 and 7.5 Hz, 2H), 2.95 (q,  $J$  = 12.0 and 4.8 Hz, 2H), 3.77 (dd,  $J$  = 11.7 and 3.3 Hz, 1H), 3.96 (dd,  $J$  = 11.5 and 4.0 Hz, 1H), 4.14 (m, 1H), 4.87 (d,  $J$  = 3.9 Hz, 1H), 7.71 (d,  $J$  = 7.5 Hz, 1H). IR (film): 3365, 2933, 1658, 1462, 1022.  $^{13}C$ -NMR (75 MHz,  $CDCl_3$ ): 7.1, 8.3, 14.2, 22.7, 25.8, 27.3, 29.3, 29.4, 29.5, 29.65, 29.68, 30.3, 31.9, 53.6, 62.6, 70.1, 107.2, 116.1, 160.3, 199.1. ESI-MS:  $m/z$  396  $[M + 1]^+$ , 418  $[M + Na]^+$ . HR-MS calcd for  $C_{23}H_{41}NO_4$ : 395.30356; found: 395.30351.

**(1'S,2'R)-N-[2-Hydroxy-1-hydroxymethyl-2-(2-tridecylcyclopropen-1-yl)ethyl]-2-oxooctanamide (6).** Yield 71% from 0.266 mmol of **10** and 2-oxooctanoic acid (0.30 mmol).  $[a]_D^{20}$ : +5.08 ( $c$  0.45,  $CHCl_3$ , 20 °C).  $^1H$ -NMR (300 MHz,  $CDCl_3$ ): 0.87 (t,  $J$  = 6.9 Hz, 6H), 1.01 (s, 2H), 1.2–1.4 (m, 30H), 1.58 (m, 4H), 2.45 (dt,  $J$  = 7.5 and 1.5 Hz, 2H), 2.90 (t,  $J$  = 7.5 Hz, 2H), 3.77 (dd,  $J$  = 11.7 and 3.9 Hz, 1H), 3.91 (dd,  $J$  = 11.4 and 3.9 Hz, 1H), 4.12 (m, 1H), 4.87 (d, 3.9 Hz, 1H), 7.70 (d,  $J$  = 8.4 Hz, 1H).  $^{13}C$ -NMR (75 MHz,  $CDCl_3$ ): 8.3, 13.9, 14.1, 22.4, 22.6, 23.1, 25.8, 27.2, 28.7, 29.34, 29.36, 29.4, 29.5, 29.64, 29.67, 31.5, 31.9, 36.8, 53.8, 62.5, 69.8, 107.2, 116.0, 160.4, 199.7. IR (film): 3365, 2926, 1672, 1530, 1461. ESI-MS  $m/z$  474  $[M + Na]^+$ , 925  $[2M + Na]^+$ . HR-MS calcd for  $C_{27}H_{49}NO_4$ : 451.36616; found: 451.36610.

**(1'S,2'R)-N-[2-Hydroxy-1-hydroxymethyl-2-(2-tridecylcyclopropen-1-yl)ethyl]-2-oxobenzamide (7).** Yield 57% from 0.266 mmol of **10** and 2-oxobenzic acid (0.30 mmol).  $[a]_D^{20}$ : +4.97 ( $c$  0.36,  $CHCl_3$ , 20 °C).  $^1H$ -NMR (300 MHz,  $CDCl_3$ ): 0.87 (t,  $J$  = 6.9 Hz, 3H), 1.04 (s, 2H), 1.2–1.4 (m, 20 H), 1.56 (t,  $J$  = 7.2 Hz, 2H), 2.46 (dt,  $J$  = 7.5 and 1.5 Hz, 2H), 3.86 (dd,  $J$  = 11.4 and 3.6 Hz, 1H), 4.01 (dd,  $J$  = 11.7 and 3.9 Hz, 1H), 4.28 (m, 1H), 4.95 (d,  $J$  = 3.9 Hz, 1H), 7.44 (t,  $J$  = 7.5 Hz, 2H), 7.62 (tt,  $J$  = 7.5 and 1.5 Hz, 1H), 7.86 (d,  $J$  = 7.5 Hz, 1H), 8.07 (d,  $J$  = 6.9 Hz, 0.5H, Har), 8.29 (dd,  $J$  = 7.8 Hz, 1.5H).  $^{13}C$ -NMR (75 MHz,  $CDCl_3$ ): 8.2, 14.0, 22.5, 25.7, 27.1, 29.2, 29.3, 29.4, 29.56, 29.58, 31.8, 53.7, 62.5, 69.8, 107.1, 116.0, 128.3, 128.4, 130.0, 131.0, 133.0, 134.4, 162.6, 187.4. IR (film): 3315, 2924, 1665, 1460, 1267, 1042. ESI-MS  $m/z$  466  $[M + Na]^+$ , 909  $[2M + Na]^+$ . HR-MS calcd for  $C_{27}H_{41}NO_4$ : 443.30356; found: 443.30359.

**(1'S,2'R)-N-[2-Hydroxy-1-hydroxymethyl-2-(2-tridecylcyclopropen-1-yl)ethyl]-12-[(5-nitro-2,1,3-benzoxazol-6-yl)amino]-dodecanamide (GT11-C12NBD).** The same procedure described for  $\alpha$ -ketoamides was applied to 0.048 mmol of **10** and 12-(5-nitro-2,1,3-benzoxazol-6-yl)aminododecanoic acid (0.050 mmol) to prepare GT11-C12NBD in 81% yield.  $^1H$ -NMR (300 MHz,  $CDCl_3$ ): 0.87 (t,  $J$  = 7.0 Hz, 3H), 1.01 (s, 2H), 1.20–1.40 (s, 34H), 1.56 (m, 2H), 1.60–1.82 (m, 4H), 2.25 (t,  $J$  = 7.5 Hz, 2H), 2.44 (dt,  $J$  = 7.5 and 1.5 Hz, 2H), 3.31 (m, 2H), 3.49 (q,  $J$  = 6.0 Hz, 2H), 3.74 (dd,  $J$  = 11.5 and 3.5 Hz, 1H), 3.83 (s, 1H), 3.90 (dd,  $J$  = 11.5 Hz and 3.5 Hz, 1H), 4.17 (m, 1H), 4.85 (s, 1H), 6.17 (d,  $J$  = 8.5 Hz, 1H), 6.42 (d,  $J$  = 7.0 Hz, 1H), 6.80 (bs, 1H), 8.50 (d,  $J$  = 8.5 Hz, 1H).  $^{13}C$ -NMR (75 MHz,  $CDCl_3$ ): 8.1, 14.1, 22.6, 24.6, 25.8, 26.1, 27.2, 27.8, 29.3, 29.3, 29.4, 29.6, 31.9, 36.0, 43.6, 53.3, 63.0, 70.6, 96.6, 98.5, 107.3, 115.7, 123.6, 136.5, 143.9, 144.2, 173.2. IR (film): 2923, 2852, 1633, 1556, 1504, 1297, 800. ESI-MS:  $m/z$  673  $[M + 1]^+$ , 696  $[M + Na]^+$ . HR-MS calcd for  $C_{37}H_{61}N_5O_6$ : 671.46218; found: 671.46208.

## Enzyme activities

**Dihydroceramide desaturase.** The effect of compounds on this enzyme was determined as reported in previous articles.<sup>12,17</sup>

**Ceramidase.** Fresh rat livers (50–60 g) were homogenized in 150 ml of 20 mM cold phosphate buffer (pH 7.4) containing 0.25 M sucrose, 1 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 1000g for 10 min, and the pellet was washed twice with 50 ml of the same buffer. After centrifugation (1000g for 10 min), the combined supernatants were centrifuged at 10000g for 30 min. The pellet of this centrifugation was resuspended and stirred for 1 h in 145 ml of 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100. The supernatant obtained after centrifugation of the mixture at 10000g for 30 min was used as a source of neutral ceramidase.<sup>20</sup>

To determine the activity of the acid ceramidase, rat livers (50–60 g) were homogenized in 150 ml of cold 50 mM sodium acetate buffer (pH 4.5) containing 0.25 M sucrose and 1 mM EDTA. The homogenate was centrifuged at 10000g for 10 min at 4 °C and the supernatant was used as a source of acid ceramidase.

The activities of both enzymes were determined following a modification of reported procedures.<sup>19</sup> Different amounts of the test compound and 3 nmol of Cer-C12NBD were dissolved in ethanol (2 µl) and the final volume was brought to 75 µl with the suitable buffer (neutral ceramidase, 25 mM tris-HCl, pH 7.4; acid ceramidase, 25 mM sodium acetate, pH 4.5, 0.25% Triton X-100). To this solution, 300–480 µg of protein was added and the suspension was incubated at 37 °C for either 1 hour (neutral) or 3 hours (acidic). The reactions were stopped by addition of either methanol (0.4 ml, neutral ceramidase) or 0.25 vol of 100 mM glycine buffer pH 10 and then methanol (0.4 ml, acid ceramidase) and the reaction mixtures were vigorously stirred and centrifuged at 5000g for 2 min. The supernatants were loaded onto a *Macro Prep High Q*, (Bio-Rad) column, which was washed sequentially with 1 ml of 25 mM tris-HCl, pH 7.5, and 1 ml of methanol–tris-HCl buffer (1 : 1) to recover the starting substrate. The enzymatically borne 12-(4-nitrobenzo-2-oxa-1,3-diazolo)dodecanoic acid was eluted with acetic acid–methanol (1 : 1) (1 ml). The fluorescence of 200 µl aliquots of each acid-eluted fraction was measured in a 96-well plate reader at excitation and emission wavelengths of 460 of 540 nm, respectively. Actual amounts of acid were calculated from a standard curve prepared by serial dilutions in the elution solvent mixture of a 30 µM solution of synthetic 12-(4-nitrobenzo-2-oxa-1,3-diazolo)dodecanoic acid.

**Kinetic parameters of hydrolysis of Cer-C12NBD and GT11-C12NBD by rat liver ceramidases.** Enzyme activities were determined as described above with 300 µg (neutral) or 480 µg (acidic) of protein. Different concentrations of the fluorescent derivatives from 10 to 160 µM were incubated with the appropriate enzyme preparation for 1 h (neutral) or 3 h (acidic) and the amounts of released acid were determined after ion exchange chromatography. The data were obtained from a representative experiment with three replicates.

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## References

- J. Ohanian and V. Ohanian, *Cell. Mol. Life Sci.*, 2001, **14**, 2053–2068.
- T. Kolter and K. Sandhoff, *Angew. Chem., Int. Ed.*, 1999, **38**, 1532–1568.
- C. Michel, G. van Echten-Deckert, J. Rother, K. Sandhoff, E. Wang and A. H. Merrill, Jr., *J. Biol. Chem.*, 1997, **272**, 22432–22437.
- P. Ternes, S. Franke, U. Zahring, P. Sperling and E. Heinz, *J. Biol. Chem.*, 2002, **277**, 25512–25518.
- F. Omae, M. Miyazaki, A. Enomoto, M. Suzuki, Y. Suzuki and A. Suzuki, *Biochem. J.*, 2004, **379**, 687–695.
- K. Bernardo, R. Hurwitz, T. Zenk, R. J. Desnick, K. Ferlinz, E. H. Schuchman and K. Sandhoff, *J. Biol. Chem.*, 1995, **270**, 11098–11102; J. Koch, S. Gartner, C. M. Li, L. E. Quintern, K. Bernardo, O. Levran, D. Schnabel, R. J. Desnick, E. H. Schuchman and K. Sandhoff, *J. Biol. Chem.*, 1996, **271**, 33110–33115; J. Bar, T. Linke, K. Ferlinz, U. Neumann, E. H. Schuchman and K. Sandhoff, *Hum. Mutat.*, 2001, **17**, 199–209.
- S. El Bawab, A. Bielawska and Y. A. Hannun, *J. Biol. Chem.*, 1999, **274**, 27948–27955; S. El Bawab, P. Roddy, T. Qian, A. Bielawska, J. J. Lemasters and Y. A. Hannun, *J. Biol. Chem.*, 2000, **275**, 21508–21513; M. Tani, N. Okino, K. Mori, T. Tanigawa, H. Izu and M. Ito, *J. Biol. Chem.*, 2000, **275**, 11229–11234; M. Tani, N. Okino, S. Mitsutake, T. Tanigawa, H. Izu and M. Ito, *J. Biol. Chem.*, 2000, **275**, 3462–3468; Y. Yoshimura, N. Okino, M. Tani and M. Ito, *J. Biochem.*, 2002, **132**, 229–236; Y. Yoshimura, M. Tani, N. Okino, H. Iida and M. Ito, *J. Biol. Chem.*, 2004, **279**, 44012–44022.
- Y. Yada, K. Higuchi and G. Imokawa, *J. Biol. Chem.*, 1995, **270**, 12677–12684.
- C. Mao, R. Xu, Z. M. Szulc, A. Bielawska, S. H. Galadari and L. M. Obeid, *J. Biol. Chem.*, 2001, **276**, 26577–26588.
- N. Okino, M. Tani, S. Imayama and M. Ito, *J. Biol. Chem.*, 1998, **273**, 14368–14373; N. Okino, S. Ichinose, A. Omori, S. Imayama, T. Nakamura and M. Ito, *J. Biol. Chem.*, 1999, **274**, 36616–36622.
- C. Mao, R. Xu, A. Bielawska, Z. M. Szulc and L. M. Obeid, *J. Biol. Chem.*, 2000, **275**, 31369–31378; C. Mao, R. Xu, A. Bielawska and L. M. Obeid, *J. Biol. Chem.*, 2000, **275**, 6876–6884.
- G. Triola, G. Fabrias and A. Llebaria, *Angew. Chem., Int. Ed.*, 2001, **40**, 1960–1962.
- J. Usta, S. El Bawab, P. Roddy, Z. M. Szulc, Yusuf, A. Hannun and A. Bielawska, *Biochemistry*, 2001, **40**, 9657–9668.
- B. Koutek, G. D. Prestwich, A. C. Howlett, S. A. Chin, D. Salehani, N. Akhavan and D. G. Deutsch, *J. Biol. Chem.*, 1994, **269**, 22937–22940.
- D. G. Deutsch, R. Omeir, G. Arreaza, D. Salehani, G. D. Prestwich, Z. Huang and A. Howlett, *Biochem. Pharmacol.*, 1997, **53**, 255–260; T. Bisogno, D. Melck, L. Depetrocellis, M. Y. Bobrov, N. M. Gretskeya, V. V. Bezuglov, N. Sitachitta, W. H. Gerwick and V. Dimarzo, *Biochem. Biophys. Res. Commun.*, 1998, **248**, 515–522; D. L. Boger, H. Sato, A. E. Lerner, B. J. Austin, J. E. Patterson, M. P. Patricelli and B. F. Cravatt, *Bioorg. Med. Chem. Lett.*, 1999, **9**, 265–270; D. L. Boger, H. Sato, A. E. Lerner, M. P. Hedrick, R. A. Fecik, H. Miyauchi, G. D. Wilkie, B. J. Austin, M. P. Patricelli and B. F. Cravatt, *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 5044–5049.
- L. De Petrocellis, D. Melck, N. Ueda, S. Maurelli, Y. Kurahashi, S. Yamamoto, G. Marino and V. Di Marzo, *Biochem. Biophys. Res. Commun.*, 1997, **231**, 82–88; S. Vandevoorde, K. Tsuboi, N. Ueda, K. O. Jonsson, C. J. Fowler, D. M. Lambert, D. G. Deutsch, N. Ueda and S. Yamamoto, *J. Med. Chem.*, 2003, **46**, 4373–4376.
- G. Triola, G. Fabrias, M. Dragusin, L. Niederhausen, R. Broere, A. Llebaria and G. Van Echten-Deckert, *Mol. Pharmacol.*, 2004, **66**, 1671–1678.
- G. Triola, G. Fabrias, J. Casas and A. Llebaria, *J. Org. Chem.*, 2003, **68**, 9924–9932.
- M. Nikolova-Karakashian and A. H. Merrill, Jr., *Methods Enzymol.*, 2000, **311**, 194–201.
- S. El Bawab, J. Usta, P. Roddy, Z. M. Szulc, A. Bielawska and Y. A. Hannun, *J. Lipid Res.*, 2002, **43**, 141–148.
- A. Bielawska, M. S. Greenberg, D. Perry, S. Jayadev, J. A. Shayman, C. McKay and Y. A. Hannun, *J. Biol. Chem.*, 1996, **271**, 12646–12654.
- M. Selzner, A. Bielawska, M. A. Morse, H. A. Rudiger, D. Sindram, Y. A. Hannun and P. A. Clavien, *Cancer Res.*, 2001, **61**, 1233–1240; L. Samsel, G. Zaidel, H. M. Drumgoole, D. Jelovac, C. Drachenberg, J. G. Rhee, A. M. Brodie, A. Bielawska and M. J. Smyth, *Prostate (N. Y., N.Y., U. S.)*, 2004, **58**, 382–393.
- M. Raisova, G. Goltz, M. Bektas, A. Bielawska, C. Riebeling, A. M. Hossini, J. Eberle, Y. A. Hannun, C. E. Orfanos and C. C. Geilen, *FEBS Lett.*, 2002, **516**, 47–52.
- N. Ueda, K. Yamanaka and S. Yamamoto, *J. Biol. Chem.*, 2001, **276**, 35552–35557.
- A. Dagan, C. Wang, E. Fibach and S. Gatt, *Biochim. Biophys. Acta*, 2003, **1633**, 161–169.